US005516645A

United States Patent [19]

Daniel et al.

[11] **Patent Number: 5,516,645**

[45] **Date of Patent: May 14, 1996**

[54] **IMMUNOASSAY ANALYTICAL ELEMENTS CONTAINING VANADIUM IV(V^{+4})IONS**

[75] Inventors: **Daniel S. Daniel**, Rochester; **David A. Hilborn**, Henrietta; **Calvin R. Messing**, Spencerport, all of N.Y.

[73] Assignee: **Johnson & Johnson Clinical Diagnostics, Inc.**, Rochester, N.Y.

[21] Appl. No.: **232,903**

[22] Filed: **Apr. 25, 1994**

[51] **Int. Cl.**[6] **G01N 33/53**

[52] **U.S. Cl.** **435/7.920**; 435/7.930; 435/7.940; 435/7.950; 435/16; 435/21; 435/28; 435/962; 435/969; 435/970; 436/518; 436/528; 436/531; 548/101; 556/42

[58] **Field of Search** 435/7.92–.95, 435/16, 21, 25–28, 810, 962, 969, 970; 436/518, 528, 531, 810, 825; 548/101; 556/42

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,039,467	8/1977	Tucker	252/300
4,228,240	10/1980	Dawson et al.	435/188
5,089,420	2/1992	Albarella et al.	436/66

OTHER PUBLICATIONS

R. Petrucci, General Chemistry: Principles and Modern Applications, Mac Millan Publishing Company (4th ed 1985) pp. 713–715 "Vanadium".

Primary Examiner—Carol A. Spiegel
Attorney, Agent, or Firm—Dana M. Schmidt

[57] **ABSTRACT**

A dry immunoassay analytical element, for assaying a ligand, comprising a support bearing:

(a) a labeled ligand zone;

(b) a spreading zone; and

(c) a receptor zone containing a fixed concentration of an immobilized receptor for the ligand and the labeled ligand and the receptor is covalently bonded to polymeric beads having a diameter in the range of 0.1 to 5 μm;

characterized in that the element contains a compound containing a vanadium IV (V^{+4}) ion and the zones can be in the same or separate layers.

25 Claims, No Drawings

IMMUNOASSAY ANALYTICAL ELEMENTS CONTAINING VANADIUM IV(V^{+4})IONS

RELATED CASES

The present case is related to U.S. Ser. No. 08/232,920 filed on the same date as the present application in the name Daniel S. Daniel, et al and entitled IMMUNOASSAY ANALYTICAL ELEMENTS CONTAINING POLYMERS CONTAINING VANADIUM IV IONS.

FIELD OF THE INVENTION

This invention relates to an immunoassay element and use thereof in an immunoassay.

BACKGROUND OF THE INVENTION

Immunoassays, which take advantage of natural immunological reactions, have found wide-spread use as analytical techniques in clinical chemistry. Because of the specificity of the reactions, they are particularly advantageous in quantifying biological analytes that are present in very low concentration in biological fluids. Such analytes include, for example, antigens, antibodies, therapeutic drugs, narcotics, enzymes, hormones, proteins, etc.

The analyte, which is the target of the assay is referred to herein as the ligand, and the labeled analyte is referred to as the labeled ligand (including immunocompetent derivatives and analogs of such ligand). Compounds which specifically recognize the ligand and the labeled ligand and react to form complexes with them are referred to herein as receptors. The receptor and the ligand or labeled ligand form a conjugate pair. Any member of the pair can function as a receptor or a ligand.

In competitive binding immunoassays, a labeled ligand is placed in competition with unlabeled ligand for reaction with a fixed amount of the appropriate receptor. Unknown concentrations of the ligand can be determined from the measured signal of either the bound or unbound (i.e. free) labeled ligand. The reaction proceeds as follows:

ligand+labeled ligand+receptor<=>ligand-receptor+labeled ligand-receptor.

Immunoassay analytical elements are known. In general, such elements comprise receptors, such as antibodies for a ligand, immobilized in a particulate layer. In addition the element usually contains a reagent system that, through interaction with a bound or unbound species, results in a signal that can be correlated to the concentration of ligand in a sample. In use, the sample is manually combined with an enzyme labeled ligand and applied to the element. After a time, a solution containing a substrate for the labeled ligand is applied to the particulate layer. The reaction with the substrate is catalyzed by the enzyme label to form a reaction product that ultimately causes a signal color to develop. The reflection density of the color can be correlated to the concentration of the ligand in the sample. Similar signal development systems are known for other known conventional labels such as radioactive tags, chromophores, fluorophores, stable free radicals, and enzyme cofactors, inhibitors and allosteric effectors.

Multilayer immunoassay elements are thin film elements which use the above described immunoassay principles to measure analytes in serum samples. In these elements, the rate of color formation is inversely correlated to the amount of analyte present. Also, the rate of color formation is

directly proportional to the activity of the drug-labeled enzyme bound to the immobilized antibody. For the immunoassays to maintain a stable calibration, none of the enzyme activity (measured rate) can be lost in any of the slides during the specified calibration period.

Frequently, immunoassay elements are supplied to customers in plastic "cartridges" containing 50 separate elements from which one element may be removed at a time as needed. The elements are stacked one on top of another so that the lower 49 elements in the cartridge all have their top surfaces covered by the element above. However, the top element in the stack has no such covering, and therefore the surface of that element is exposed to environmental factors to which the other 49 elements are not. For example, the top (or first) element is more exposed to air flow and light than the remainder of the elements when the cartridges are being handled during manufacturing or when the cartridges are in the element supplies of the clinical analyzers.

During storage, prior to use, the cartridges themselves are stored in sealed, foil-lined bags. However, the top element is still more exposed to the residual air and humidity inside the sealed bags than the other 49 elements.

It has been found that, when a common test fluid was reacted with the elements in a cartridge, the rate of color formation observed in the top (or first) element was always lower than the rate of color formation observed when the same test fluid was applied to elements below the top element in the same cartridge.

SUMMARY OF THE INVENTION

The objective of the present invention is to substantially eliminate the lower rate of color formation of the first element in the top element of the cartridge. This objective is substantially accomplished by providing a dry immunoassay analytical element, for assaying a ligand, comprising a support bearing:

(a) a labeled ligand zone;

(b) a spreading zone; and

(c) a receptor zone containing a fixed concentration of an immobilized receptor for the ligand and the labeled ligand and the receptor is covalently bonded to polymeric beads having a diameter in the range of 0.1 to 5 μm;

characterized in that the element contains, preferably in the spreading zone or layer, a compound containing a vanadium IV (V^{+4}) ion and the zones can be in the same or separate layers.

The coverage of the vanadium IV (V^{+4}) ion in the element should be in the range 0.025 to 0.5 mmoles/m^2.

The element defined above substantially reduces the lower rate of color development of the top element in a cartridge compared to the other elements in the same cartridge. Moreover all elements in the cartridge exhibit greater long term keeping. The examples establish that any compound containing a vanadium IV (V^{+4}) ion provides these benefits.

In addition the compounds containing vanadium IV ions are useful in stabilizing enzyme compositions generally, particularly horseradish peroxidase and conjugates in which such enzymes are used as labels. For example the compounds can be used in solution assays as well as in the dry elements of this invention.

The present invention also provides a method for the assay of an immunologically reactive ligand in an aqueous liquid sample, comprising the steps of:

A. providing a dry immunoassay analytical element according to the present invention;

B. contacting a finite area of the top zone or layer of the element with a sample of the liquid sample thereby forming (i) an immobilized ligand-receptor complex, (ii) an immobilized enzyme labeled ligand-receptor complex; or (iii) a mixture of (i) and (ii).

C. contacting the finite area with a substrate solution thereby catalyzing the development of a color; and

D. determining the concentration of the ligand colorimetrically.

DETAILS OF THE INVENTION

The elements of this invention comprise labeled ligand, spreading and receptor zones. The various zones can be in one coated layer or in separate coated layers. For example the spreading zone and the receptor zone can be in a single layer or they can be in separate layers. The separate layers can be arranged in any order on the support. Or the separate layers can be arranged such that the receptor layer is directly on the support, the spreading layer directly above the receptor layer and the labeled ligand zone over the spreading layer. When the receptor zone forms an entirely separate layer, the layer will also include a binder of the type describe hereinafter. The element can include additional layers such as those described infra. All such layers can be coated using coating techniques known in this art and which are briefly described infra.

The labeled ligand zone or layer may be gravure coated to 1) minimize wet coverage of the labeled ligand coating composition, to avoid precontact of the labeled ligand with the receptor, while at the same time maintaining enough wetness to achieve uniform coverage of the labeled ligand, and 2) achieve rapid drying in a way that a) removes substantially all of the coating solvent; b) avoids adversely affecting the porosity of the spread layer and spreading time, and c) maintains sufficient enzyme activity.

The relative affinity of antibody and labeled ligand for each other is also an important factor in minimizing prebinding. This factor is controlled, as is well known by those skilled in this art, by manipulating the structure of the labeled ligand together with a prudent choice of antibody.

In general the level of coated labeled ligand coverage needed in an element is determined empirically for each specific immunoassay according to the following procedure:

1. Determine the concentration of labeled ligand needed to achieve acceptable immunoassay performance when the immunoassay is performed by contacting the analytical element with the labeled ligand concurrently with a sample. Acceptable assay performance is achieved when (a) the assay can be carried out in less than 20 minutes; (b) the dynamic range of the assay is such that the minimum and maximum ligand concentrations detectable cover a clinically useful concentration range; and (c) clinically significant ligand concentrations can be detected across the dynamic range.
2. Empirically determine the level of coated labeled ligand coverage needed with the same analytical element to achieve the above established acceptable assay performance by:
   A. Coating, directly over the particulate receptor layer of the element used to establish optimum spotted labeled ligand levels, the labeled ligand at a coverage in g/m^2 that is some fraction, multiple or the same as

the concentration of labeled ligand used in spotting the labeled ligand in 1, supra.

B. Conduct a series of assays with test samples containing a known concentration of the ligand.

C. Compare the results of the assays with the known concentration of ligand; and

D. Repeat steps B and C as needed, varying the labeled ligand coverage according to the results seen in step 2C to determine the labeled ligand coverage required.

Depending on the labeled ligand, the coverage of the labeled ligand could be less than, the same as or several multiples greater (2×, 3×, 4×, etc.) than the labeled ligand concentration needed when the same assay is carried out by spotting the labeled ligand directly on the analytical element.

Using the above guidelines, carefully controlled gravure coating procedures were successfully carried out using the following coverages and drying protocols. The labeled ligand coatings in the elements of the invention were prepared with a gravure machine (made by Yasui of Japan). Drying conditions used for all of the examples were 120° F. (49° C.) in the first drying section only. The second section was not used. The gravure cylinder used contained 295 cells/inch (1.344×10^8 cells/m^2). The cells had a depth of 19 microns, a width of 72 microns and a land width between cells of 12 microns. This cylinder will deliver about 4.3 g/m^2 of coating composition containing the labeled ligand to the bead spreading layer using the direct gravure process at a coating machine speed of 50 ft/min (15.24 m/minute). Those skilled in the gravure coating arts will be readily able to adapt the previously described procedure to any gravure coating machine. The coating composition for the labeled ligand was as follows:

Coated Labeled Ligand Coating Composition
Based on 4.3 g/m^2 Wet Coverage

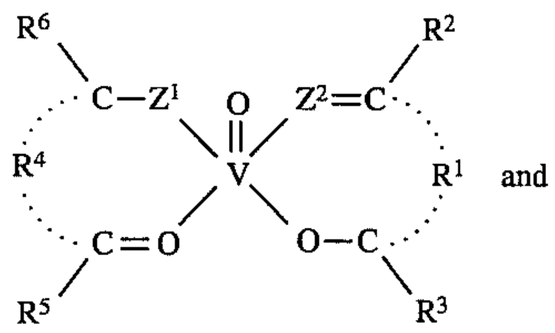
Component	g/m^2 Dry Coverage
MOPS Buffer (3-[N-morpholino]propane sulfonic acid)	.0045
BSA (Bovine Serum Albumin)	.000215
poly(acrylamide)	.00108
4'-Hydroxyacetanilide	.000325
*Labeled ligand	.000016

*Labeled ligand has been coated anywhere between 4 and 64 μg/m^2

The remaining layers of the element can be coated using well known coating techniques in this art. To minimize prebinding each layer is coated separately and allowed to dry before application of subsequent layers.

The spreading layer or zone is porous and coated over the receptor zone or layer. It contains, as an essential ingredient, the vanadium IV ion. Representative compounds containing such ions are vanadyl (VO^{+2}) containing compounds such as vanadyl salts such as vanadyl sulfate; β-diketone or α-ketol complexes defined by the following structural formulas I–V:

Useful vanadyl β-diketone complexes have the structure (I):



I

and

wherein

R^1 and R^4, each independently, represent methylidyne (=CH—) or a single covalent bond;

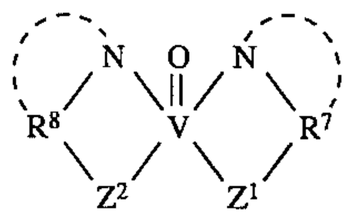
R^2, R^3, R^5, and R^6 each independently represent, straight or branched, substituted or unsubstituted alkyl of about 1 to 6 carbon atoms; substituted or unsubstituted aryl of about 6 to 14 carbon atoms; and substituted or unsubstituted heterocyclic groups having 5 or 6 carbon atoms and heteroatoms in the ring such as 2-furyl, 2-thienyl, pyranyl, thiopyranyl wherein the substituents are alkyl (1–6 carbons), haloalkyl, e.g., trifluoromethyl, tribrommethyl, and pentachloroethyl; aralkyl such as benzyl and phenethyl; aryl of 6–10 carbons such as phenyl, tolyl, xylyl chlorophenyl, bromophenyl and naphthyl; hydroxy; and halo such as chloro or bromo; stopped here

or R^1, R^2 and R^3 or R^4, R^5 and R^6 taken together with the carbon atoms to which they are attached represent a substituted or unsubstituted, unsaturated carbocyclic group wherein the substituents are alkyl, aryl, and halo; an unsaturated heterocyclic group having 5 to 6 carbon and hetero atoms in the ring including such groups having one or two additional fused aromatic rings such as 4H-pyran, 2-methyl-4H-pyran, 2-phenyl-4H-benzopyran, 2H-pyran, 4H-thiopyran, 2-methyl-4H-thiopyran, 2-phenyl-4H-benzothiopyran;

and Z^1 and Z^2, each independently, represent oxy (—O), thio (—S—) or imino (—NH—):

provided that when R^1 is a single covalent bond, R^1, R^2, and R^3 are taken together as described, or when R^4 is a single covalent bond, R^4, R^5, and R^6 are taken together as described. The complex of vanadyl and 4-(2-furyl)-1,1,1-trifluorobutane-2-4-dione is an example of structure I.

Useful vanadyl (VO+2) containing compounds can have a structure II:



II

wherein;

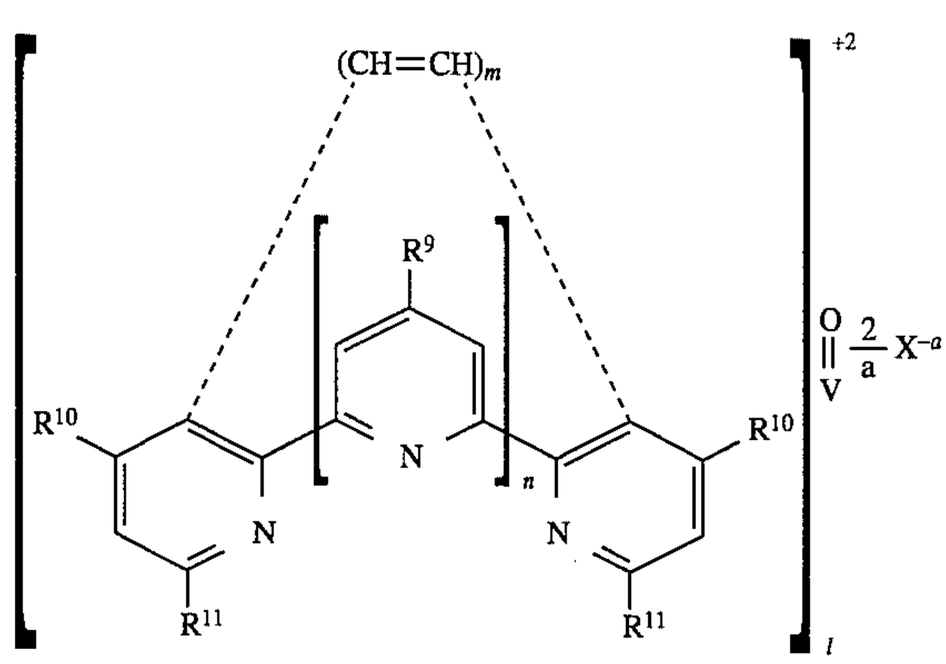
Z^1 and Z^2, each independently, represent oxy (—O—), thio (—S—), or imino (—NH—);

R^7 and R^8, each independently, taken together with the nitrogen atom to which each is attached, represent the atoms necessary to complete, a substituted or unsubstituted heterocyclic group having 5 to 6 nuclear atoms; or such heterocylic group having 1 to 3 additional fused aromatic rings, such as benzo, naphtho and phenanthro groups having rings containing 6 carbon atoms each;

provided that Z^1 and Z^2 are appended to carbon atoms that are separated from the nitrogen atoms of their respective heterocyclic group by no more than three additional carbon or hetero atoms of the type described previously.

Embodiments of structure II contemplated include compounds wherein Z^1 is appended to an aromatic ring fused to the heterocylic group formed with R^7; Z^2 is appended to an aromatic ring fused to the heterocylic group formed with R^8; and the heterocylic groups are, each independently, unsubstituted or substituted with a substituent selected from the group consisting of halo, alkyl, or aryl. Heterocylic groups are substituted or unsubstituted quinoline, acridine, naphtho [1,2-h]quinoline, or benzo[h]quinoline. The vanadyl complex of 8-hydroxyquinoline is an example of a useful structure II compound.

Useful vanadyl (VO+2) containing compounds can have a structure III:



III

wherein:

R^9 represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, aryl, or aryloxy;

R^{10} represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, or substituted or unsubstituted aryl;

R^{11} represents carboxy, hydroxy, carbonylnitrilodiacetic acid, methylenenitrilodiacetic acid, hydrazinylylidene diacetic acid or the salts of such acids;

X^{-a} is an acid anion;

a is 1, 2, or 3;

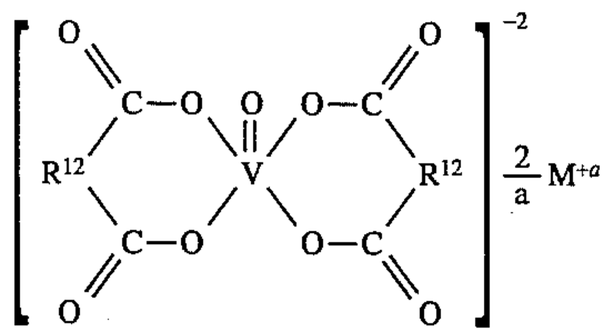
1 is 2 or 3;

m is 0 to 1; and

n is 0 to 4;

provided that m is 1 only when n is 0.

Useful vanadyl (VO+2) containing compounds can have a structure IV:



IV

wherein:

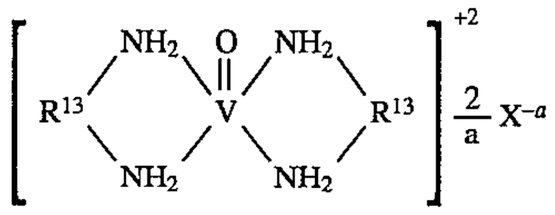
each R^{12} independently represents a substituted or unsubstituted 1,2- or 1,3-arylene of 6 to 14 nuclear carbon atoms; substituted or unsubstituted 1,2-, 1,3-, or 1,4-alkylene of about 2 to 10 carbon atoms, including alkylene interrupted with hetero atoms —O—, —S—, —NH;

M^{+a} is a metal or ammonium cation; and

a is 1, 2, or 3.

Compounds in which R^{12} is 1,2-dihydroxyethylene are examples of a structure IV compound e.g., the vanadyl complex of tartaric acid.

Useful vanadyl (VO+2) containing compounds can have a structure V:



V

wherein:

R^{13} each independently represents a substituted or unsubstituted 1,2- or 1,3-arylene of 6 to 14 nuclear carbon atoms; or substituted or unsubstituted 1,2-, 1,3-, or 1,4-alkylene of about 2 to 10 carbon atoms, including alkylene interrupted with hetero atoms —O—, —S—, —NH—; and

X^{-a} is an acid anion;

a is 1, 2, or 3.

Compounds in which R^{13} is ethylene, iminodiethylene, or oxydiethylene are examples of structure V compounds.

In structures I–V alkyl is about 1 to 6 carbon atoms such as methyl, ethyl, isopropyl, butyl,. hexyl, trifluoromethyl, tribromomethyl, and pentachloroethyl. Alkyl substituents and alkyl in alkoxy, alkylthio, alkylamino and alkylene are similarly defined. Alkyl substituents also include halo (Cl, Br, Fl and I) and hydroxy. Aryl is about 6 to 14 carbon atoms such as phenyl, naphthyl, tolyl, xylyl, chlorophenyl, bromophenyl, nitrophenyl, hydroxyphenyl, tolyl, xylyl, methoxyphenyl, methylthiophenyl, and carboxyphenyl. Aryl substituents are alkyl as defined above, hydroxy and halo (Cl, Br, Fl and I). Aryloxy, aralkyl and arylene are similarly defined.

Other materials for use in spreading layers are well known in the art of making dry analytical elements as disclosed, for example, in U.S. Pat. No. 4,258,001. Such layers include macroporous layers made from cloth, paper, etc. A preferred particulate layer is a bead spreading layer (BSL). This layer can be easily constructed to have suitable porosity for use in the elements of the present invention to accommodate a test sample (e.g. 1 to 100 μL), diluted or undiluted. Preferably, the spreading layer is isotropically porous, which property is created by interconnected spaces between the particles comprising the zone. By isotropically porous is meant that the spreading layer uniformly spreads the applied fluid in all directions throughout the layer.

Useful spreading layers, including bead spreading layers are disclosed in U.S. Pat. Nos. 4,670,381; 4,258,001 and 4,430,436. Particularity useful spreading layers are those having a particulate structure formed by organo-polymeric particles and a polymeric adhesive for those particles described in U.S. Pat. No. 4,258,001. The organo-polymeric particles useful in the spreading layer are generally heat-stable, spherical beads having a particle size in the range of from about 10 to 40 μm in diameter or even smaller.

The particles can be composed of a wide variety of organic polymers, including both natural and synthetic polymers, having the requisite properties. Preferably, however, they are composed of one or more addition polymers described in the aforementioned patents.

When the receptor layer is a separate layer it is prepared and coated over a support. The receptors are covalently bonded to polymer particles through surface reactive groups on the receptor (nucleophilic free amino groups and sulfhydryl groups).

A general procedure for attaching receptors to the small polymer beads includes covalently attaching the selected receptor to the beads using generally known reactions. With many pendant groups for example the haloalkyl, 2-substituted activated ethylsulfonyl and vinylsulfonyl, the receptor can be directly attached to the beads. Generally, the beads are mixed with the receptor in an aqueous buffered solution (pH generally from about 5 to about 10) and a concentration of from about 0.1 to about 40 weight percent polymer particles (preferably from about 0.1 to about 10 weight percent). The amount of receptor is at a ratio to polymer of from about 0.1:1000 to about 1:10, and preferably from about 1:100 to about 1:10. Mixing is carried out at a temperature in the range of from about 5° to about 50° C., and preferably at from about 5° to about 40° C., for from about 0.5 to about 48 hours. Any suitable buffer can be used.

In some instances, the pendant reactive groups on the outer surface must be modified or activated in order to cause covalent attachment of the ligand. For example, carboxyl groups must be activated using known carbodiimide or carbamoylonium chemistry, described in EP 308235 published 22 Jul. 1992 and U.S. Pat. No. 5,155,166.

The attachment of the receptor to carboxyl group-containing monodispersed polymer beads, however, is carried out in two steps, the first of which involves contacting an aqueous suspension of the particles with a carbodiimide or a carbamoylonium compound to produce reactive intermediate polymer particles having intermediate reactive groups in place of the carboxyl groups. This step is carried out at a suitable pH using suitable acids or buffers to provide the desired pH. Generally, the pH is less than 6, but this is not critical as long as the reaction can proceed. More likely, the pH is between about 3.5 and about 7. The molar ratio of carbodiimide or carbamoylonium compound to the carboxyl groups on the surface of the particles is from about 10:1 to 500:1.

In the second step of the method, the reactive intermediate formed in the first step is contacted with a reactive amine- or sulfhydryl-group containing receptor. A covalent linkage is thereby formed between the particles and the receptor. The weight ratio of the receptor to the polymeric particles is generally from about 1:1000 to about 1:1, and preferably from about 1:100 to about 1:10.

In other instances, an epoxy group on the outer surface can be hydrolyzed to form a diol compound capable of reacting with cyanogen bromide which can act as a coupling agent for amine groups in the immunological species. Aldehydes can react directly with amines to form a Schiff's base which can be subsequently reduced to form a covalent link. Alternatively, the aldehyde can be oxidized to an acid and chemistry identified above for carboxyl groups can be used to form an amide linkage.

Any reactive amine- or sulfhydryl-containing receptor can be attached to the monodispersed polymeric beads as long as that receptor contains a reactive amine or sulfhydryl group, respectively which will react with the reactive groups on the polymer or with the intermediate formed by the reaction of a carbodiimide or a carbamoylonium compound with carboxyl groups on the particles in the case which the polymer has reactive carboxyl groups.

The small polymer beads having reactive groups that readily react directly with the amine or sulfhydryl groups on the receptors are simply mixed with the receptors, in an appropriate buffer if necessary, and allowed to react.

Polymers from which beads for the receptor can be selected include the following: poly(m & p-chloromethylstyrene), poly(styrene-co-m & p-chloromethylstyrene-co-2-hydroxyethyl acrylate) (67:30:3 molar ratio), poly(styrene-co-m & p-chloroethylsulfonylmethylstyrene) (95.5:4.5 molar ratio), poly{styrene-co-N-[m & p-(2-chloroethylsulfonylmethyl)phenyl]acrylamide} (99.3:0.7 molar ratio),

poly(m & p-chloromethylstyrene-co-methacrylic acid) (95:5, 98:2 and 99.8:0.2 molar ratio), poly (styrene-co-m & p-chloroethylsulfonylmethyl-styrene-co-methacrylic acid) (93.5:4.5:2 molar ratio), poly{styrene-co-N-[m & p-(2-chloroethylsulfonylmethyl)phenyl]acrylamide-co-methacrylic acid} (97.3:0.7:2 molar ratio), poly(styrene-co-m & p-chloromethylstyrene) (70:30 molar ratio), poly[styrene-co-3-(p-vinylbenzylthio)propionic acid] (97.6/2.4 molar ratio), poly (styrene-co-vinylbenzyl chloride-co-acrylic acid) (85:10:5 molar ratio), Poly(styrene-co-acrylic acid) (99:1 molar ratio), poly(styrene-co-methacrylic acid) (90:10 molar ratio), poly(styrene-co-acrylic acid-co-m & p-divinylbenzene) (89: 10:1 molar ratio), poly(styrene-co-2-carboxyethyl acrylate) (90:10 molar ratio), poly (methyl methacrylate-co-acrylic acid) (70:30 molar ratio), poly (styrene-co-m & p-vinylbenzaldehyde) (95:5 molar ratio), and poly(styrene-co-m & p-vinylbenzaldehyde-co-methacrylic acid)(93:5:2 molar ratio).

The element is carried on a suitable support. The receptor layer is coated over the support although there may be intervening layers, such as a gelatin/buffer layer, between the support and the receptor layer. The support can be any suitable dimensionally stable, and preferably, nonporous and transparent (i.e. radiation transmissive) material which transmits electromagnetic radiation of a wavelength between about 200 and about 900 nm. A support of choice for a particular element should be compatible with the intended mode of detection (reflection, transmission or fluorescence spectroscopy). Useful support materials include polystyrene, polyesters [e.g. poly(ethylene terephthalate)], polycarbonates, cellulose esters (e.g. cellulose acetate), etc.

Polymeric binders for the receptor layer are described generally in Canadian patent 1,240,445 and are expressly incorporated herein by reference. Useful polymers are chill-gellable polymers comprising from about 30 to 97 weight percent of polymerized N-alkyl substituted acrylamide such as N-isopropylacrylamide. Other useful N-alkyl-substituted acrylamides include N-n-butylacrylamide, N,N-diethylacrylamide and N-n-propylacrylamide. Poly(N-isopropylacrylamide-co-methacrylic acid-co-N,N'-methylenebisacrylamide is used in the examples to illustrate the utility of these binders.

The polymer binder also comprises from about 3 to 25 weight percent of one or more polymerized crosslinking monomers having at least two addition-polymerizable groups per molecule. These crosslinking monomers are generally well known in the art. The preferred crosslinking monomers contain acrylamido or methacrylamido groups to facilitate polymerization with the N-alkyl-substituted acrylamides.

Examples of useful crosslinking monomers include:

N,N'-methylenebisacrylamide;
N,N'-methylenebismethacrylamide;
ethylene dimethacrylate;
2,2-dimethyl-1,3-propylene diacrylate;
divinylbenzene;
mono[2,3-bis(methacryloyloxy)propyl]phosphate;
N,N'-bis(methacryloyl)urea;
triallyl cyanurate;
allyl acrylate;
allyl methacrylate;
N-allylmethacrylamide;
4,4'-isopropylidenediphenylene diacrylate;
1,3-butylene diacrylate;
1,4-cyclohexylenedimethylene dimethacrylate;
2,2'-oxydiethylene dimethacrylate;
divinyloxymethane;
ethylene diacrylate;
ethylidene diacrylate;
propylidene dimethacrylate;
1,6-diacrylamidohexane;
1,6-hexamethylene diacrylate;
1,6-hexamethylene dimethacrylate;
phenylethylene dimethacrylate;
tetramethylene dimethacrylate;
2,2,2-trichloroethylidene dimethacrylate;
ethylenebis(oxyethylene) diacrylate;
ethylenebis(oxyethylene) dimethacrylate;
ethylidyne trimethacrylate;
propylidyne triacrylate;
vinyl allyloxyacetate;
1-vinyloxy-2-allyloxyethane;
2-crotonoyloxyethyl methacrylate;
diallyl phthalate; and
2-(5-phenyl-2,4-pentadienoyloxy)ethyl methacrylate.

These chill gellable polymeric binders can also include 0 to 60 weight percent of polymerized hydrophilic monomers. Amounts of 5 to 35 weight percent are also useful. Hydrophilic monomers are disclosed in Canadian patent 1,240,445. In particular such monomers have one or more groups selected from hydroxy, pyrrolidone, amine, amide, carboxy, sulfo, carboxylate salt, sulfonate salt and sulfate salt groups. Generally the counter ions of the salt groups are alkali metal or ammonium. Useful hydrophilic monomers are acrylic acid and methacrylic acid and their salts, 2-acrylamido-2-methylpropane sulfonate, 2-hydroxyethyl acrylate, 2-hydroxyethyl methacrylate, 2-hydroxypropyl acrylate, 2-hydroxypropyl methacrylate and glyceryl methacrylate.

Further, the recited binders make it possible to form uniform coatings of receptor layers due to the very low binder viscosities achieved from sheer thinning during extrusion hopper coating. A further advantage is achieved with the recited binders in that, immediately after forming uniform coatings, the viscosity of the binders increases substantially resulting in a "set layer"that remains stable and uniform during wet transport and drying of the binders.

The receptors can also be dispersed in a polymer binder selected from the group consisting of:

poly(vinyl alcohol);
bovine serum albumin;
acacia gum;
homopolymers of poly-N-vinylpyrrolidone having a molecular weight in the range 8000 to 400,000; and
water-soluble vinyl addition copolymers having two or more monomers selected from the group consisting of acrylamide, methacrylamide, N-alkyl substituted acrylamides, N-alkyl substituted methacrylamides, 1-vinylimidazole, 2-alkyl substituted-1-vinylimidazoles, 2-hydroxyalkyl substituted 1-vinylimidazoles, N-vinylpyrrolidone, hydroxyalkyl acrylates, hydroxyalkyl methacrylates, acrylic acid, and methacrylic acid; wherein alkyl and hydroxyalkyl in the copolymers has 1 to 6 carbon atoms such methyl ethyl, propyl and hexyl.

The element can comprise one or more additional layers, e.g. separate or combined reagent/spreading layer and a

gelatin/buffer layer containing other necessary additives such as electron transfer agents.

The gelatin/buffer layer or the reagent layer or the spreading layer of the element can contain the indicator composition comprising one or more reagents dispersed in one or more synthetic or natural binder materials, such as gelatin, or other naturally-occurring colloids, homopolymers and copolymers, such as poly(acrylamide), poly(vinylpyrrolidone), poly(N-isopropylacrylamide), poly(acrylamide-co-N-vinyl-2-pyrrolidone) and similar copolymers. The indicator composition can also be dispersed in the receptor layer.

Other optional layers, e.g., subbing layers, radiation-blocking layers, etc. can be included if desired. All layers of the element are in fluid contact with each other, meaning that fluids and reagents and uncomplexed reaction products in the fluids can pass between superposed regions of adjacent layers.

The layers of the element can contain a variety of other desirable but optional components, including surfactants, thickeners, buffers, hardeners, antioxidants, coupler solvents, and other materials

known in the art. The amounts of these components are also within the skill of a worker in the art.

The elements can be used to determine low concentrations of immunologically reactive ligands in a liquid, such as a biological fluid (e.g., whole blood, serum, plasma, urine, spinal fluid, suspensions of human or animal tissue, feces, saliva, lymphatic fluid and the like). The ligands can be determined at concentrations as low as about 10^{-15} molar, and most generally at a concentration of from about 10^{-11} to about 10^{-4} molar.

Ligands which can be so determined, either quantitatively or qualitatively, include therapeutic drugs (e.g., phenobarbital, digoxin, digitoxin, theophylline, gentamicin, quinidine, phenytoin, propanolol, carbamazepine, tobramycin, lidocaine, procainamide and the like), natural or synthetic steroids (e.g., cortisol, aldosterone, testosterone, progesterone, estriol, etc.), hormones (e.g., thyroid hormones, peptide hormones, insulin, etc.), proteins (e.g. albumin, IgG, IgM, ferritin, blood clotting factors, C-reactive protein, isoenzymes, apolipoproteins, etc.), antigens, antibodies including monoclonal antibodies, and other species which will naturally react with a receptor. This invention is particularly useful for the determination of therapeutic drugs, such as digoxin, phenytoin, carbamazepine, theophylline, or phenobarbital and hormones such as thyroxine or triiodothyronine.

The assay can be carried out using any enzyme label which can be attached to the ligand to form a labeled ligand. Enzymes, such as glucose oxidase, peroxidases such as horseradish peroxidase (HRP), alkaline phosphatase and galactosidase are preferred labels.

It is within the skill of the ordinary worker in clinical chemistry to determine a suitable substrate for a given label. The substrate can be a material which is directly acted upon by the enzyme label, or a material that is involved in a series of reactions which involve enzymatic reaction of the label. For example, if the enzyme label is a peroxidase, the substrate is hydrogen peroxide plus an appropriate reducing agent. Using glucose oxidase as an example, the substrate glucose is generally present in the reagent layer or added as a substrate solution to yield about 0.01 moles/m^2, and preferably from about 0.001 to about 0.1 mole/m^2. A worker skilled in the art would know how to adjust the amount of a particular substrate for the amount of enzyme label used in the assay.

The reagent layer may contain an indicator composition comprising one or more reagents which provide a detectable species as a result of the reaction catalyzedby the label. The detectable species could develop a color, be radioactive, fluoresce, or be chemiluminescent. For present purposes the invention is illustrated using a colorimetric indicator composition which provides a colorimetrically detectable species as a result of enzymatic reaction of an enzyme-labeled ligand analog with a substrate.

The indicator composition can be a single compound which produces a detectable dye upon enzymatic reaction, or a combination of reagents which produce the dye. For example, when glucose is used as the substrate and glucose oxidase as the enzyme label, the colorimetric indicator composition can include a coupler and an oxidizable compound which react to provide a dye, Alternatively, the composition can include a leuco dye and peroxidase or another suitable peroxidative compound which generates a detectable dye as a result of the formation of hydrogen peroxide produced when glucose oxidase converts glucose to gluconic acid. Useful leuco dyes are known in the art and include those, for example, described in U.S. Pat. No. 4,089,747 (issued May 16, 1978 to Bruschi) and U.S. Ser. No. 612,509, filed May 21, 1984 by Babb et al. The particular amounts of the colorimetric indicator composition and its various components are within the skill of a worker in the art.

The labeled ligands can be prepared using known starting materials and procedures, or obtained commercially. Generally, the ligand is attached to the label (e.g. an enzyme moiety) through a covalent bond.

The immunoassay can be manual or automated. In general, the amount of a ligand in a liquid is determined by taking the element from a supply roll, chip packet or other source and physically contacting a finite area of the spreading layer with a sample of the liquid, e.g. 1 to 100 μL. The finite area which is contacted is generally no more than about 150 mm^2.

The amount of ligand is determined by passing the element through a suitable apparatus for detecting the complexed ligand analog directly or the detectable species formed as a result of enzymatic reaction of an enzyme label and a substrate. For example, the species can be detected with suitable spectrophotometric apparatus using generally known procedures. In an enzymatic reaction, the resulting product is determined by measuring, for example, the rate-of change of reflection or transmission density in the finite area which was contacted with the test sample. The area which is measured has a diameter of generally from about 3 to about 5 mm. The amount of ligand in the liquid sample is inversely proportional to the amount of label measured in the finite area. Generally, label measurement is made after application of a substrate solution.

A typical element having a spreading layer in which vanadium IV (V^{+4}) is present, as a vandyl salt or a vandyl β-diketone complex, is included in the working examples is presented below.

Comparative Example 1

An element, free of vanadium IV (V^{+4}), for conducting an immunoassay of phenytoin in serum samples was prepared on a poly(ethylene terephthalate) support. The element had the following configuration and ingredients.

Layer	Material	Dry Coverage (Grams/Meter^2)
Gravure	Label	16×10^{-6}
	Magenta Dye	0.0269
	MOPS Buffer, pH 7.0	0.0045
	BSA	2.15×10^{-4}
	Polyacrylamide	1.08×10^{-3}
	TX-100 ™ surfactant	4.3×10^{-3}
	Trehalose	0.215
Bead Spread Layer	TES Buffer, pH 7.0	0.219
	Dimedone	0.45
	4'-Hydroxyacetanilide	0.45
	Mannitol	1.0
	BSA	1.0
	Glycerol	2.0
	Adhesive Polymer	2.58
	Polymer Beads (20-40 μM)	130.0
Receptor Layer	Polymer Binder	0.60
	Leuco Dye	0.20
	Dimedone	0.05
	Tetronic T908 ™ surfactant	0.02
	Olin 10G ™ surfactant	0.01
	TES Buffer, pH 7.0	0.10
	TX-100 ™ surfactant	0.02
	Antibody Beads	0.15
Gel	Gelatin	10.0
	4'-Hydroxyacetanilide	0.15
	TES Buffer, pH 7.0	4.58
	TX-100 ® surfactant	0.020
	BVSME	0.150

The components listed in the above element are as follows:

Label: The phenytoin-amine-enriched horseradish peroxidase label is a conjugate of an amine-enriched horseradish peroxidase and a phenytoin hapten having an extended linking chain.

Amine-enriched HRP was prepared as follows. Dry HRP was dissolved in 0.1 M MES buffer, pH 5.5, to achieve a final concentration of 2.5×10^{-6} mol (100 mg) in 10 mL of buffer (MES=2-(N-morpholino)ethanesulfonic acid). The protein concentration was determined by A_{403} measurement using the conversion factor A_{403} 1 mg/mL=2.24. The HRP solution was combined with 1.5×10^{-3} mol (275 mg) of L-lysine monohydrochloride dissolved in 10 mL of 0.1M MES buffer at pH 5.5. A solution of freshly prepared 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 5×10^{-4} mol, 960 mL) in MES buffer was added. The container was capped and mixed overnight at room temperature. The reaction was dialyzed against 0.02M MOPS buffer at pH 7.0 (3 L at 10° C.). The dialysis buffer was changed 3×. MOPS=3-(N-morpholino)propanesulfonic acid.

Prior to reaction, a sample of the amine enriched HRP was exchanged from MOPS buffer into 0.1M EPPS buffer, pH 8.0, using 30,000 NMWL (nominal molecular weight limit cutoff) Centricells centrifugal ultrafilters. This sample was then diluted to obtain a solution with a final concentration of 5.71 mg/mL.

Meanwhile the phenytoin hapten 5,5-diphenyl-3-{4-[4-(3-succimimidoxycarbonylpropionyl)-1-piperazonylcarbonyl]butyl}-2,4-imidazolidene (described in preparative example 2 of U.S. patent application Ser. No. 712,330 filed Mar. 16, 1992 and which application is expressly incorporated herein by reference) (15.5 mg) was dissolved in 1.031 mL of dry DMF containing 10 mM of 4'-hydroxyacetanilide. The resulting solution (500 μL) was added slowly while stirring to a solution of 1 mL of the above amine-enriched HRP in EPPS buffer in 500 μL of 10 mM 4'-hydroxyacetanilide (4'-HA) in DMF. The molar ratio of the phenytoin/HRP was 50/1.

Incubation was performed for 1 hour with gentle shaking in a 42° C. water bath. The sample was transferred to Spectropor #2 dialysis tubing and dialyzed as follows:

a) 1 L DMF 4'-HA/0.1M EPPS, pH8.0 (1:1) at 41° C. for 1 hour;

b) Dialysis condition a) was repeated 1 time;

c) 1.5 L 0.1M EPPS, pH 8.0, containing 0.1% bovine serum albumin at 8° C. for 1.5 hour;

d) 1.5 L 0.1M EPPS, pH 8.0, at 8° C. for 18 hours;

e) 1.5 1)0.04M TRIS HCl/0.15M NaCl, pH 7.5, 8° C. for 2 hours; and

f) Dialysis condition e) was repeated 1 time for 4 hours.

Following dialysis, 0.02% merthiolate was added as a preservative, and the labels were stored refrigerated.

Magenta Dye: 4,5-Dihydroxy-3-(6,8-disulfo-2-naphthylazo)-2,7-naphthalenedisulfonic acid, sodium salt (KAN 905783).

MOPS: 3-(N-Morpholino)propanesulfonic acid buffer.

EPPS: 3-[4-(2-hydroxyethyl-1-piperazinyl]propanesulfonic acid buffer.

BSA: Bovine serum albumin.

TX-100: Triton X-100 surfactant—an octylphenoxy polyethoxy ethanol surfactant sold by Union Carbide.

TES: N-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid buffer.

Adhesive Polymer: Poly(methyl acrylate-co-sodium 2-acrylamido-2-methylpropanesulfonate-co-2-acetoacetoxyethyl methacrylate).

Polymer Beads: Poly (vinyltoluene-co-methacrylic acid) particles having an average diameter in the range of 20–40 μM.

Polymer Binder: Poly(N-isopropylacrylamide-co-methacrylic acid-co-N,N'-methylenebisacrylamide).

Leuco Dye: 4,5-Bis(4-dimethylaminophenyl)-2-(3,5-dimethoxy-4-hydroxyphenyl)imidazole.

Tetronic T908: A nonionic surfactant which is a block copolymer of ethylene oxide and propylene oxide sold by BASF Corp.

Olin 10G: An isononylphenoxypolyglycidol surfactant averaging about 10 glycidol units per molecule sold by Olin Chem. Co.

Antibody Beads: Polymer particles of poly[styrene-co-p-(2-chloroethylsulfonylmethyl)styrene] having an antibody to phenytoin covalently bound thereto.

BVSME: Bis(vinylsulfonylmethyl) ether.

DTPA: Diethylenetriaminepentaacetic acid.

Cartridges containing these elements were prepared and placed on a laboratory benchtop overnight. The light in the laboratory was left on overnight. The next day, the elements were tested in a prototype automated thin-film immunoassay analyzer. Ten uL of a test solution containing 10 ug/mL phenytoin was applied to an element. The element was then incubated for 5 minutes at 37° C. after which 10 uL of a wash fluid containing Na_2HPO_4 (10 mM, pH 6.8), 4'-hydroxyacetanilide (5 mM), hexadecylpyridinium chloride (0.1%), H_2O_2 (8.8 mM), and DTPA (10 uM) was applied to the slide. This fluid both washes away unbound label from the detection area and initiates the HRP catalyzed color formation reaction. The element was then placed in a 37° C. incubator and reflectance density readings were taken every 3 seconds at 670 nM. The rate of color formation was calculated from these readings. The results were:

Rate of Top element (n = 1)*	Rate on Non-Top elements (n = 10)	% Rate Loss of Top elements
0.0731 Dt/Min	0.0867 Dt/Min	–16%

The top slide had a significantly lower rate than the other elements in the cartridge.

*n=The number of elements tested. The Dt/Min value is the average for the number of slides tested.

Comparative Example 2

The lower rates observed in the top elements could also be induced in lower elements by removing the elements below the top elements from the cartridges and placing them on the benchtop, thereby increasing their exposure to environmental factors (for example, light, air) to which the top slides in cartridges were subjected. This resulted in an even greater loss of rates then observed in the top slide in a cartridge.

Phenytoin elements of the formulation described in comparative Example 1 were placed on a laboratory benchtop overnight. Cartridges containing similar slides were also prepared and also placed on the same laboratory benchtop overnight. The light in the lab was left on during the experiment. The next day, the elements were tested in a prototype automated thin-film immunoassay analyzer as described in comparative Example 1. The results were (the rates from the top elements in cartridges were not included in the data analysis):

Rate of Benchtop elements (n = 10)	Rate of Cartridge elements (n = 10)	% Rate Loss of Benchtop Elements
0.0325 Dt/Min	0.0867 Dt/Min	–63%

Although this type of exposure was far more severe than encountered by the top element in a cartridge, it provided a convenient experimental system to study the effect of environmental factors on rate of color development.

EXAMPLE 1

The low rate of color formation of the top element would cause the analyte sample tested on this element to be incorrectly predicted. In order to alleviate this situation, the formulation of the elements were improved to prevent environmental factors from having this adverse affect on the top element. It was found that the incorporation of vanadyl bis(acetylacetonate) in coatings caused the rate of color development in elements placed overnight on the benchtop to be much more resistant to exposure to the environment.

Phenytoin elements were prepared using the formulation of comparative Example 1 except that vanadyl bis(acetylacetonate) was incorporated into the bead spread layer at a dry coverage of 0.052 g/m^2.

In an experiment similar to that described in comparative example 2, elements of this new formulation were placed directly on the laboratory benchtop overnight and cartridges containing these new elements were prepared, and the cartridges were also placed on the benchtop overnight. The light in the laboratory was left on overnight. The next day, the elements were tested in a prototype automated thinfilm immunoassay analyzer as described in comparative example 1. The results were (the rates from the top elements in cartridges were not included in the data analysis):

Rate of Benchtop elements (n = 10)	Rate of Cartridge elements (n = 10)	% Rate Loss of Benchtop Elements
0.0693 Dt/Min	0.0759 Dt/Min	–9%

The 9% rate loss using the new formulation was much improved compared to the 63% rate loss using the original formulation.

EXAMPLE 2

Top elements in cartridges were also less susceptible to loss of rate when prepared using this new formulation. In an experiment similar to the one described in comparative Example 1 (top element in a cartridge compared to non-top elements in the same cartridge), the results were:

Rate of Top element (n = 1)	Rate of Non-Top elements (n = 10)	% Rate of Top elements
0.0762 Dt/Min	0.0759 Dt/Min	+0.5%

In this experiment, the percent rate of the top element compared to the non-top slides (+0.5%) was improved compared to the original formulation (Example 1, –16%).

EXAMPLE 3

In addition to reducing the sensitivity of the top element to environmental effects, the vanadyl bis(acetylacetonate) (VO(acac)2) was also found to enhance longer term room temperature stability of stored phenytoin analytical elements. Cartridges containing phenytoin elements of the original formulation and other cartridges containing phenytoin elements of the improved formulation were prepared and placed in a covered tray in a room whose temperature was controlled at 25° C. and whose humidity was controlled at 37% RH for four weeks (stored cartridges). During this same period of time, control cartridges of the two formulations were kept in a freezer (–20° C.). At the end of the four weeks, the elements in the control cartridges (after equilibrating to room temperature) and the stored cartridges were tested using a prototype automated thin-film immunoassay analyzer as described in comparative Example 1 except that an additional phenytoin test solution containing 20 ug/mL phenytoin was included in the test. The rate from the top element in each cartridge was excluded from the data analysis. The results were:

Formula	Test Fluid	Rate of Control elements (Dt/Min)	Rate of Stored elements (Dt/Min)	% Rate Retained (Rate Stored/ Rate Control) × 100
Original	10 ug/mL	0.0752 (n = 15)	0.0583 (n = 15)	78
	20 ug/mL	0.0529 (n = 5)	0.0440 (n = 5)	83
Orig. +	10 ug/mL	0.0758 (n = 14)	0.0725 (n = 15)	96
VO (acac) 2	20 ug/mL	0.0501 (n = 5)	0.0535 (n = 5)	1.07

The addition of vanadyl bis(acetylacetonate), hereafter $VO(acac)_2$, to the bead spread layer in the element improved long term stability of the element.

Dry coverages of $VO(acac)_2$ between 13 and 52 mg/m^2 do not affect the above results significantly. Higher dry coverage was not tested because of solubility concerns of $VO(acac)_2$ in the bead spread layer. Below 13 mg/m^2 of $VO(acac)_2$ degradations in the improvement were apparent although improvements over the comparative examples were still evidenced. Although phenytoin was used in the examples, equivalent improvements have been observed for phenobarbital and digoxin.

EXAMPLES 4–6

An analytical element of the following formulation was prepared on a poly(ethylene terephthalate) support:

Layer	Material	Dry Coverage (Grams/Meter^2)
Bead Spread Layer	TES Buffer, pH 7.0	0.219
	Adhesive Polymer	2.58
	Polymer Beads (20–40 mM)	130.0
Receptor Layer	Polymer Binder	0.60
	Leuco Dye	0.20
	Dimedone	0.05
	Tetronic T908	0.02
	Olin 10G	0.01
	TES Buffer, pH 7.0	0.10
	TX-100	0.02
Gel	Gelatin	10.0
	4'-Hydroxyacetanilide	0.15
	TES Buffer, pH 7.0	4.58
	TX-100	0.020
	BVSME	0.150

This analytical element was used to measure HRP stability with the following protocol. Ten microliters of a solution of 10 mM sodium phosphate buffer, pH 7.0, containing about 3×10^{-8}M HRP was spotted onto each of three elements. These elements were placed in a dark drawer overnight. The next day, the elements were removed from the drawer and the HRP was extracted by immersing each element in 1 mL of a solution of 10 mM sodium phosphate, 0.15M sodium chloride, 0.1% bovine serum albumin, pH 7.0, in a test tube to extract the HRP. After vortexing the test tube (which removed the analytical element components from the poly(ethylene terephthalate) support), the resulting suspension was centrifuged and the solution removed. The amount of active HRP in this solution was determined by taking a 100 μL aliquot and adding this aliquot to a spectrophotometer cuvette already containing 800 μL of a solution of 10 mM sodium phosphate, pH 6.8, 10 μM diethylenetriaminepentaacetic acid, 5 mM 4'-hydroxyacetanilide, 1.25% polyvinylpyrrolidone, 0.01% 4,5-bis(4-dimethylaminophenyl)-2-(4-hydroxy-3-methoxyphenyl)imidazole leuco dye and 8.8 mM hydrogen peroxide, and 100 μL of a solution of 5 mM 4'-hydroxyacetanilide. A blue color was formed whose rate was determined spectrophotometrically at 670 nm. The rate of color formation was directly proportional to the amount of active enzyme in the extract.

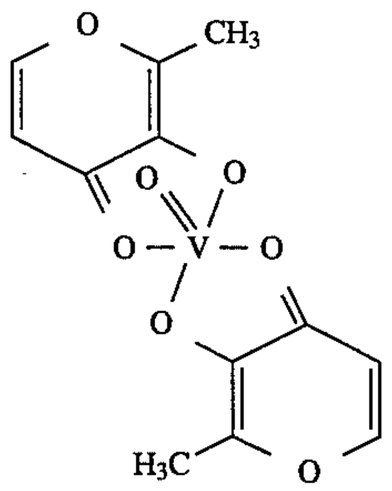
In the case described above (the HRP being applied to the elements in a solution of 10 mM phosphate buffer, pH 7.0), the amount of active enzyme extracted from the elements after the above protocol was followed was compared to the amount of active enzyme applied to the element (fraction active HRP extracted).

The above part of the experiment was the control. It was repeated 4 times except that one of the compounds of the invention listed in the data table was added to the HRP-phosphate buffer solution used to spot each element before dark storage at a level of 1 mM. The results are provided in the following table:

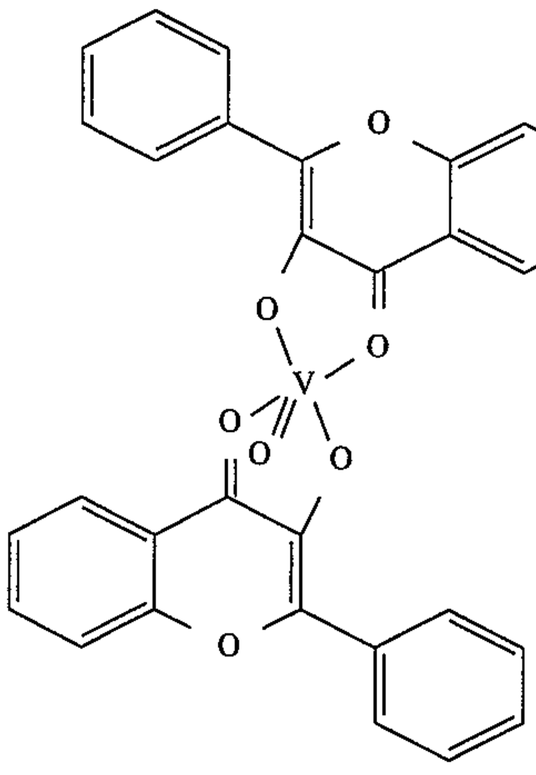
Example	Compound Added to the HRP Solution	Fraction of Active HRP Extracted	% HRP Activity Retained
	Control (None)	0.24	24%
4	Vanadyl Sulfate ($VOSO_4$)		67%
5	Vanadyl Complex of 3-Hydroxy-2-methyl-4H-pyran-4-one	0.48	48%
6	Vanadyl Complex of 3-Hydroxyflavone	0.41	41%
7	Vanadyl Complex of 8-Hydroxyquinoline	0.64	64%

The percent of HRP activity retained when compounds of the invention were present (41–67%) was much improved when compared to the control containing no compounds of the invention (24%). The structures of the compounds of Examples 5 to 7 are as follows:

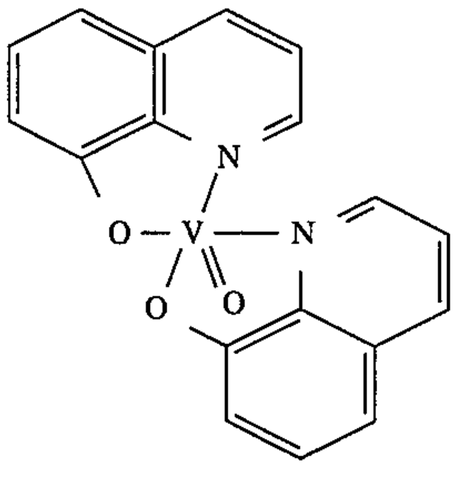
Example 5



Example 6



Example 7



EXAMPLES 8–11

In these examples, analytical elements for the detection of the therapeutic drug phenobarbital were used. These analytical elements had a composition similar to the phenytoin slides described in comparative Example 1 except that the antibody beads had an antibody to phenobarbital covalently bound to them. The label was a phenobarbital/amine-enriched horseradish. The gelatin layer contained 3',5'-dichloro-4'-hydroxyacetanilide instead of 4'-hydroxyacetanilide. The label was prepared as described in U.S. patent applications Ser. Nos. 851,435 and 851,436, both filed Mar. 16, 1992, and expressly incorporated herein by reference.

The bead spread layer contained none (control) or one of the following vanadium IV-containing compounds:

Example	Vanadyl Compound	Coverage (mg/m^2)
8	Vanadyl bis (acetylacetonate) (Acetylacetonate)	26
9	Vanadyl complex of 1-(2-thienyl)-4,4,4-trifluorobutane-1,3-dione (Thenyl)	51
10	Vanadyl complex of 1-(2-furyl)-4,4,4-trifluorobutane-1,3-dione (Furyl)	48
11	Vanadyl complex of tartaric acid (Tartrate)	52

The above coverages all correspond to a vanadyl (VO) coverage of 6.7 mg/m^2.

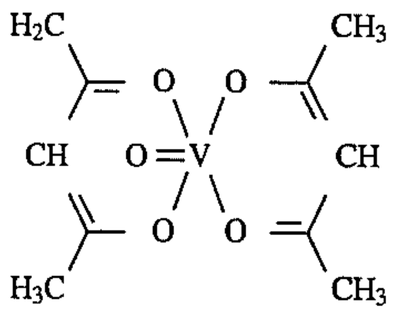
In an experiment similar to that described in comparative Example 2, phenobarbital elements of the new formulations were placed directly on the laboratory benchtop overnight. Cartridges containing elements of the new formulations (3 cartridges of each formulation) were prepared and also placed on the benchtop overnight. The light in the laboratory was left on overnight. The next day, the elements were tested in a prototype automated immunoassay analyzer as described in comparative Example 1. Five elements, in addition to the top element from each of the three cartridges containing identical formulations, were tested so that the total number of "cartridge" elements tested was 15. The results were (the rates from the top element in cartridges were not included in the data analysis):

Example	Vanadyl Compound	Rate (Dt/Min) of Benchtop elements (n = 10)	Rate (Dt/Min) of Cartridge elements (n = 15)	% Rate Loss of Benchtop elements
Control	None	0.0118	0.0725	–84
8	Acetylacetonate	0.0573	0.0737	–22
9	Thenyl	0.0488	0.0595	–18
10	Furyl	0.0466	0.0655	–29
11	Tartrate	0.0482	0.0667	–28

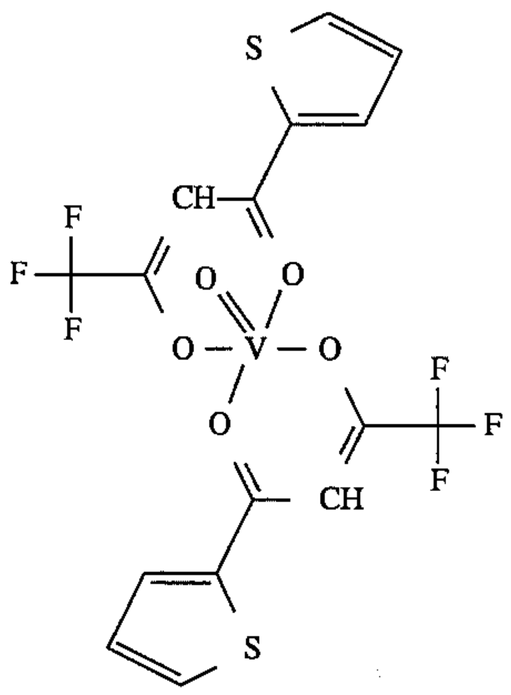
The 18% to 29% rate loss using the vanadyl compounds was much improved compared to the 84% rate loss in the slides containing no vanadyl compound.

Top elements in the cartridges were also less susceptible to loss of rate if they contained these other vanadyl compounds. In an experiment similar to the one described in comparative example 2 except that 3 cartridges of each formulation were left out overnight. The 3 top elements of each cartridge were tested. Five non-top elements were sampled from each cartridge for a total of 15 non-top elements. The results were:

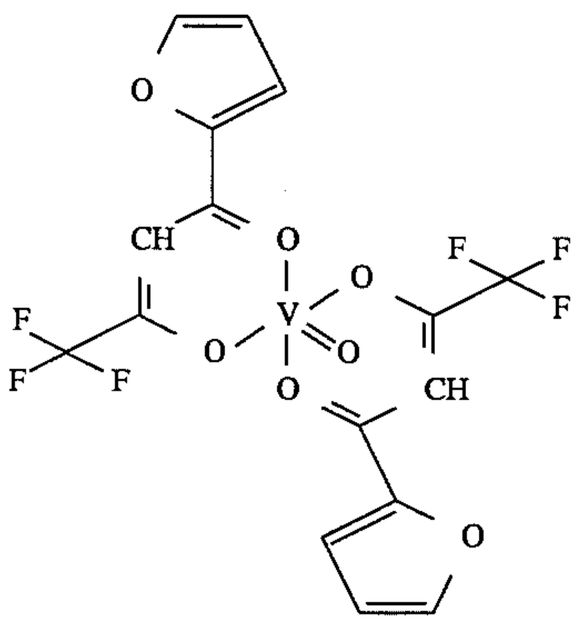
Example 8



Example 9



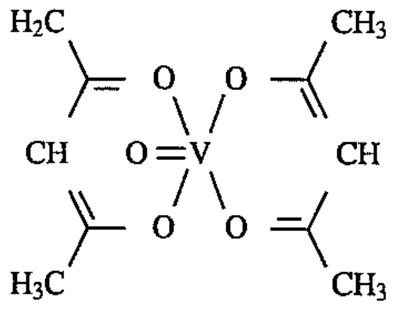
Example 10



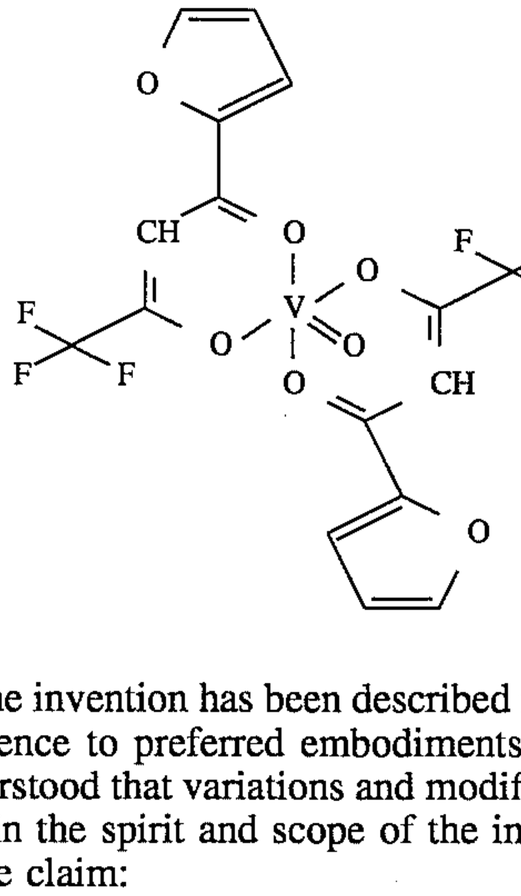
This 2% to 3% rate loss using the vanadyl compounds was much improved compared to the 49% rate loss in the elements without the vanadyl compounds.

The compounds of Examples 8–10 have the structures:

Example 8



Example 9



Example 10

The invention has been described in detail with particular reference to preferred embodiments thereof, but it will be understood that variations and modifications can be effected within the spirit and scope of the invention.

We claim:

1. In a dry immunoassay analytical element, for assaying a ligand, comprising a support bearing:

a) a labeled ligand zone containing an enzyme-labeled ligand;

b) a spreading zone; and

c) a receptor zone containing a fixed concentration of an immobilized receptor for the ligand and the labeled ligand wherein the receptor is covalently bonded to polymeric beads having a diameter in the range of 0.1 to 5 um;

the improvement wherein the spreading zone comprises a compound containing a vanadium IV (V^{+4}) ion, said compound being a) compatible with said receptor and said labeled ligand to permit said immunoassay to take place and b) selected from the group consisting of vanadyl and vanadite compounds,

said compound being further present in said element within a range of from 0.025 to 0.5 mmoles per m^2,

said zones being in one or more separate layers.

2. The element of claim 1 wherein the vanadium IV (V^{+4}) is present as a vanadyl (VO^{+2}) containing compound.

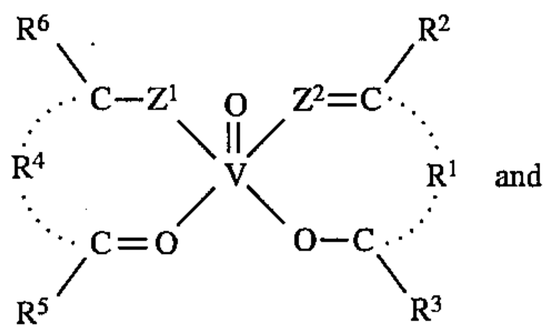
3. The element of claim 1 wherein vanadium IV (V^{+4}) ion is present in a compound selected from the group consisting of vanadyl salts and vandyl B-diketone complexes.

4. The element of claim 3 wherein the vanadium IV (V^{+4}) compound comprises a vanadyl complex of acetylacetone.

5. The element of claim 4 wherein the vanadium IV (V^{+4}) ion is present in vanadyl bis(acetylacetonate).

6. The element of claim 3 wherein the vanadyl salt is vanadyl sulfate.

7. The element of claim 3 wherein the vanadyl β-diketone complex has structure (I):



I

and

wherein

R^1 and R^4, each independently, represent methylidyne (=CH—) or a single covalent bond;

R^2, R^3, R^5, and R^6 each independently represent, straight or branched, substituted or unsubstituted alkyl of about 1 to 6 carbon atoms; substituted or unsubstituted aryl of about 6 to 14 carbon atoms; and substituted or unsubstituted t0 heterocyclic groups having 5 or 6 carbon and heteroatoms in the ring;

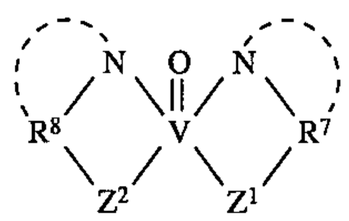
or R^1, R^2 and R^3 or R^4, R^5 and R^6 taken together with the carbon atoms to which they are attached represent a substituted or unsubstituted, unsaturated carbocyclic group of one or two rings wherein the substituents are alkyl of 1 to 6 carbon atoms, aryl of 6 to 14 carbon atoms, and halo; an unsaturated heterocyclic group having 5 to 6 carbon and hetero atoms in the ring; and

Z^1 and Z^2, each independently represent oxy, thio or imino;

provided that when R^1 is a single covalent bond, R^1, R^2, and R^3 are taken together as described, or when R^4 is a single covalent bond, R^4, R^5, and R^6 are taken together as described.

8. The element of claim 7 wherein the structure I compound is a vanadyl complex of 1-(2-furyl)-4,4,4-trifluorobutane-1,3-dione.

9. The element of claim 2 wherein vanadyl (VO+2) is present in a compound having structure II:



II

wherein;

Z^1 and Z^2, each independently, represent oxy (—O—), thio (—S—), or imino (—NH—);

R^7 and R^8, each independently, taken together with the nitrogen atom to which each is attached , represent the atoms necessary to complete, a substituted or unsubstituted heterocyclic group having 5 to 6 nuclear atoms; 1 to 3 rings containing 6 carbon atoms each;

provided that Z^1 and Z^2 are appended to carbon atoms that are separated from the nitrogen atoms of their respective heterocyclic group by no more than three additional carbon or hetero atoms.

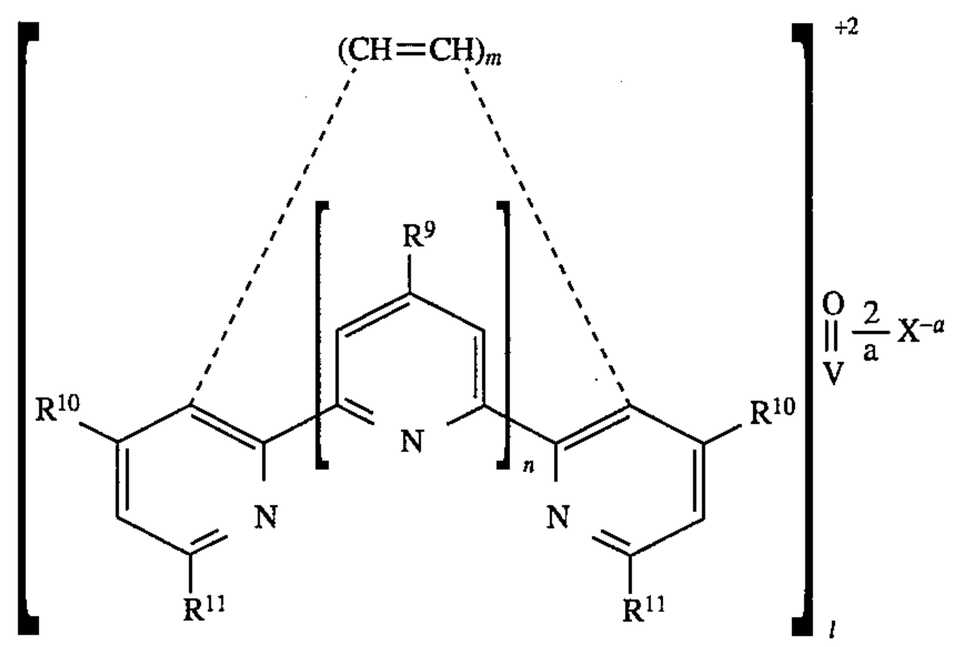
10. The element of claim 9 wherein Z^1 is appended to an aromatic ring fused to the heterocylic group formed with R^7; Z^2 is appended to an aromatic ring fused to the heterocylic group formed with R^8; and the heterocylic groups are, each independently, substituted with a substituent selected from the group consisting of halo, alkyl, or aryl.

11. The element of claim 10 wherein the heterocylic groups are substituted or unsubstituted quinoline, acridine, naphtho[1,2-h]quinoline, or benzo[h]quinoline.

12. The element of claim 11 wherein the heterocylic group is quinoline.

13. The element of claim 12 wherein the compound is a vanadyl complex of 8-hydroxyquinoline.

14. The element of claim 2 wherein the vanadyl (VO^{+2}) is present in a compound having structure III:



III

wherein:

R^9 represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, aryl, or aryloxy;

R^{10} represents hydrogen, alkyl, alkoxy, alkylthio, alkylamino, or substituted or unsubstituted aryl;

R^{11} represents carboxy, hydroxy, carbonylnitrilodiacetic acid, methylenenitrilodiacetic acid, hydrazinylylidene diacetic acid or the salts of such acids;

X^{-a} is an acid anion;

a is 1, 2, or 3;

1 is 2 or 3;

m is 0 to 1; and

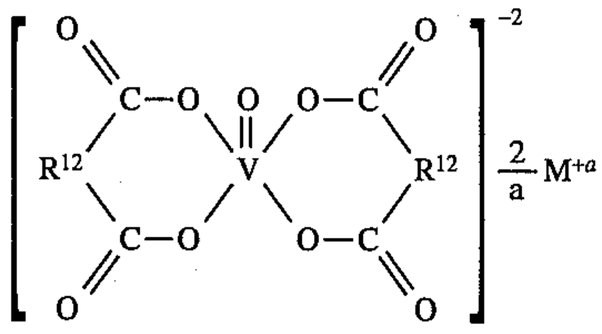
n is 0 to 4;

provided that m is 1 only when n is 0.

15. The element of claim 14 wherein alkyl, alkoxy, alkythio, and alkyamino wherein are each straight or branched having 1 to 6 carbon atoms, aryl and aryloxy are substituted or unsubstituted and have 6 to 14 carbon atoms.

16. The element of claim 15 wherein X is sulfate.

17. The element of claim 2 wherein the vanadyl (VO^{+2}) is present in a compound having structure IV:



IV

wherein:

each R^{12} independently represents a substituted or unsubstituted 1,2- or 1,3-arylene of 6 to 14 nuclear carbon atoms; or substituted or unsubstituted 1,2-, 1,3-, or 1,4-alkylene of about 2 to 10 carbon atoms, including alkylene interrupted with hetero atoms —O—, —S—, and —NH—;

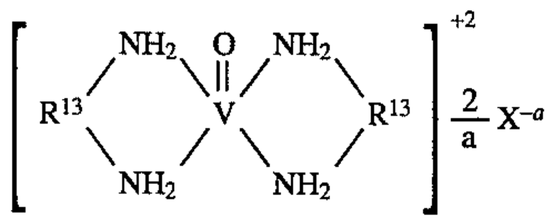
M^{+a} is a metal or ammonium; and

a is 1, 2, or 3.

18. The element of claim **17** wherein R^{12} is 1,2-dihydroxyethylene.

19. The element of claim **18** wherein the compound is a vanadyl complex of tartaric acid.

20. The element of claim **2** wherein the vanadyl (VO^{+2}) is present in a compound having structure V:



V

wherein:

R^{13} each independently represents a substituted or unsubstituted 1,2- or 1,3-arylene of 6 to 14 nuclear carbon atoms; or substituted or unsubstituted 1,2-, 1,3-, or 1,4-alkylene of about 2 to 10 carbon atoms, including alkylene interrupted with hetero atoms selected from the group consisting of —O—, —S—, —NH—;

X^{-a} is an acid anion; and

a is 1, 2, or 3.

21. The element of claim **20** wherein R^{13} is ethylene, iminodiethylene, or oxydiethylene.

22. The element of claim **2** wherein the coverage of the vanadium IV (V^{+4}) ion in the element is in the range 0.025 to 0.5 mmoles/m^2.

23. The element of claim **1** wherein the label in the labeled ligand is an enzyme.

24. A method for the assay of an immunologically reactive ligand in an aqueous liquid sample, comprising the steps of:

a.) providing a dry immunoassay analytical element according to claim **1**;

b.) contacting a finite area of the top zone or layer of the element with a sample of the liquid sample thereby forming with the sample, a mixture comprising (i) an immobilized receptor-ligand complex, (ii) an immobilized enzyme labeled ligand-receptor complex; or (iii) a mixture of (i) and (ii);

c.) contacting the finite area with a substrate solution that is reactive with said enzyme to form a color;

d.) Separating enzyme-labeled ligand that is not immobilized, from that which is immobilized; and

e.) determining the concentration of the immobilized ligand colorimetrically.

25. The element of claim **1** wherein horseradish peroxidase is the label in the labeled ligand.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : **5,516,645**

DATED : **May 14, 1996**

INVENTOR(S) : **Daniel S. Daniel, et al**

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

Column 23, Line 29, after "stituted", delete "t0".

Column 23, Line 47, after "vanadyl", delete "(VO+2)" and insert --(VO^{+2})-- therefor.

Column 24, Line 46, after "alkyamino", delete "wherein".

Signed and Sealed this

Tenth Day of September, 1996

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks